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Analysis of MRP1 and MRP3 expression profile in liver and spleen of hyperbilirubinemic rodents

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Abstract:

Unconjugated bilirubin (UCB) is produced mostly in the spleen from the turnover of heme. UCB is conjugated by the activity of hepatic UGT1A1enzyme (UDP-glucuronosyltransferase 1A1) and exported into the bile. High serum level of UCB is toxic to cells, and its intracellular accumulation is suggested to be limited by multidrug resistance proteins 1 and 3 (MRP1 and MRP3) that export UCB out of the cells to prevent its toxicity. In this study, the expression profile for MRP1 and MRP3 was analysed by qRT-PCR and Western blot in the liver and the spleen of two animal models of hyperbilirubinemia (β-thalassemic mouse and Gunn rat). The results indicated that MRP1 mRNA and protein expression remained unchanged in the liver and spleen of both animal models. MRP3 mRNA and protein are up-regulated in the spleen of β-thalassemic mouse and liver of Gunn rat. In conclusion, high level of UCB induces only the expression of MRP3 which may indicate its possible role in preventing UCB toxicity.

Keywords: Multidrug resistance-associated proteins, hyperbilirubinemia, β-thalassemia, Gunn rat.

الملخص:

البيليروبين غير المباشر هي مادة صفراء اللون تنتج عن تكسير جزيء الهيم الموجود في الهيموغلوبين المكوِّن لكريات الدّم الحمراء في الطحال، نتيجة نشاط إنزيمان هما: (Heme oxygense I and Biliverdin reductase)، نظرا ولأن مادة البيليروبين غير المباشر هي مادة غير ذائبة في الماء، فإنها تنتقل إلى الكبد وتتّحد مع حمض (Glucurionic acid) بواسطة الإنزيم (UGT1A1)، وهكذا يتحوّل الى البيليروبين المباشر الذائب في الماء، والذي يتخلّص منه الجسم عن طريق القنوات الصفراء.

قد تؤدّي بعض الحالات إلى إرتفاع نسبة البيليروبين غير المباشر في الدم عن مستوياته الطبيعية ، كما هو الحال في اليرقان الفسيولوجي (neonatal jaundice)، عندما ترتفع نسبة البيليروبين في الأيام الأولى بعد الولاده مباشرة قبل أن تعود الى مستوياتها الطبيعية. هذا الإرتفاع الفسيولوجي مهم لحماية خلايا الجسم، حيث تعتبر مادة البيليروبين من المواد القوية المضادة للأكسدة والإلتهابات. في المقابل، هناك بعض الحالات الّتي ترتفع فيها نسبة مادة البيليروبين بشكل كبير كما هو الحال مثلا في (β-Thalassemia)، حيث يؤدي تكسير كريات الدّم الحمراء باستمرار إلى إرتفاع نسبة البيليروبين غير المباشر، ومتلازمة (Crigler Najjar Syndrom Type I)، حيث يؤدي غياب الانزيم (UGT1A1) الى تراكم البيليروبين غير المباشر في الدّم. هذا الإرتفاع الكبير في مادة البيليروبين غير المباشر يحفّز عملية الموت المبرمج للخلايا (Apoptosis)، خاصّة الخلايا العصبيّة حيث يؤدّي إلى اليرقان النّووي والتّلف العقلي (Kernicterus). لأنّ جميع خلايا الجسم لا تحتوي على إنزيم (UGT1A1) ما عدا خلايا الكبد، فإنّها تستخدم وسيلة أخرى للحفاظ على مستويات قليلة من البيليروبين غير المباشر داخلها. هناك الكثير من الدراسات العلمية والتّجارب العمليّة على الخلايا والنّماذج الحيوانيّة ركزّت على دور النّقل النّشط من خلال نواقل (MRP1 and MRP3) في حماية الخلايا من سميّة مادة البيليروبين غير المباشر.

في هذه الدراسه العلمية قمنا بتحليل التعبير الجيني والبروتيني لهذه النواقل، بإستخدام (qRT-PCR and Western blot) في أنسجة الكبد والطحال، باعتبارهما الأعضاء الرئيسة المسؤولة عن عمليات أيض البليروبين في النّماذج الحيوانيّة (β-Thalassemic mouse and Gunn rat).

تشير النتائج إلى أنّ التّعبير الجيني والبروتيني للناقل (MRP1) لا تتغّير بارتفاع نسبة البيليروبين في الكبد والطحال لأي من النماذج الحيوانيه المدروسه ، ممّا يؤشّر على الدّور المحدود لهذه المضخّات في حماية الخلايا من سميّة البيليروبين غير المباشر ، في المقابل تشير النّتائج إلى أن البيليروبين غير المباشر يؤدّي إلى تحفيزالتّعبير الجيني والبروتيني للناقل (MRP3) في الكبد للنّموذج الحيواني (Gunn rat)، والطّحال للنموذج الحيواني (β-Thalassemic mouse) ، مما يدُل على الدّور المهم لهذه المضخّات في حماية الخلايا من سميّة البيليروبين غير المباشر.

Introduction:

Bilirubin is the oxidative end product of the heme catabolism. Heme degradation is performed by the enzyme heme-oxygenase I, producing biliverdin which is then converted to unconjugated bilirubin (UCB) by the biliverdin reductase. Once released in the blood and due to its poor aqueous solubility, UCB is tightly but reversibly binds to albumin and transferred into the liver where it is conjugated with glucuronic acid by the enzyme UDP-glucuronosyltransferase 1A1 (UGT1A1) and finally secreted into the bile canaliculus by the MRP2 transporter [1].

UCB has a vital role under physiological and pathological conditions. Neonatal jaundice is characterised by mild serum UCB level "physiological hyperbilirubinemia", and the pigment plays an essential cytoprotective role by acting as a potent antioxidant and anti-inflammatory molecule [2,3]. However, when the plasma level of UCB have excessively elevated the unbound or free fractions (Bf) of the UCB increase and may cause cell toxicity through different molecular mechanisms that involved oxidative stress, ER stress, calcium signalling, inflammation and apoptosis [4,5]. UCB elevation is associated with several conditions such as; low blood pH (acidosis), the use of drugs that compete with UCB for binding to plasma albumin (e.g., sulfonamide), hyperhemolysis (e.g., β-thalassemia), or defect in UGT1A1 activity (e.g., Crigler Najjar syndrome type I) [6,7].

Unlike hepatocytes, all cells do not possess conjugation activity, and must, therefore, even oxidise or export UCB to prevent its toxicity. MRPs are family of ATP-binding cassette transporters (ABC transporters) that mediate the ATP-dependent export of many physiological and xenobiotic compounds [8,9], including UCB [10].

Early studies about a possible role of MRP1 in UCB transport revealed that UCB at clinically relevant concentration is a substrate for YCF1 and YLL015 (homology to human MRP1 and MRP2, respectively) gene products of transporter mutants in *Saccharomyces cerevisciae*[11]. Pascolo et al. demonstrated that UCB undergoes ATP-dependent export from trophoblastic BeWo cells by MRP1[12]. Cultured mouse astrocytes rapidly up-regulated the expression of MRP1 when exposed to UCB [13]. Furthermore, Using Mouse Embryo Fibroblasts (MEF) of MRP1 knockout mice (-/-) and their wild-type (+/+), Calligaris et al., added more evidence that MRP1 has a functional role in protecting cells from UCB toxicity [14].

MRP3 share 58% of homology to MRP1 and studies showed it modulation by conjugated and unconjugated hyperbilirubinemia. Liver MRP3 is up-regulated in animal models of obstructive cholestasis and when the activity of MRP2 is absent as a compensatory mechanism in which the conjugated bilirubin and other substrates are exported from hepatocytes back to the bloodstream for renal excretion [15,16]. In the liver of Gunn rats (the animal model of Crigler Najjar Syndrom type I), MRP3 is induced in response to high level of UCB [17,18].

In the present study, the expression profile of MRP1 and MRP3 mRNA and protein was characterised in liver and spleen of two different models of hyperbilirubinemia; the β-thalassemic mouse with chronic hemolytic hyperbilirubinemia [19], and the Gunn rat with chronic non-hemolytic hyperbilirubinemia [20].

Materials and methods

Animals and serum bilirubin measurement: The study plan was approved by the Local Committee for Care and Use of Laboratory Animals and all animals were received human care. Adult WT (C57BL/6J) and β-thalassemic mouse models (n=5 at six months age) were received from the Department of Clinical and Experimental Medicines, University of Verona, Italy [19]. Seventeen days old (P17) of Control (NN) and homozygous (jj) Gunn rats were born and raised in the animal facility of the University of Trieste (n=5)[20]. Under anaesthesia, animals were decapitated. The livers and spleens were removed rapidly, rinsed extensively with ice-cold PBS and then snap-frozen in ice and stored at -80°C. The blood was collected, and then the total serum bilirubin concentrations were measured by diazo-reaction (Boehringer–Mannheim Kit 1552414, Monza, Italy).

Total RNA extraction and quantification: Total RNA was isolated from tissues using the TRI Reagent (Sigma, USA) following the manufacturer's instructions. RNA concentration was obtained spectrophotometrically (Beckman Coulter, DU®730) from A260 measurements (A260/A280 ratio was between 1.7 and 2.1). The integrity of the sample was assessed with 1% agarose gel electrophoresis.

Reverse Transcriptase and quantitative real-time PCR: The cDNA was obtained from 1µg of purified MMLV-derived reverse transcriptase system kit (iScript ™cDNA Synthesis Kit, BioRad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. Reverse transcriptase was performed in a thermal cycler at 25 °C for 5 min, 45 °C for 30 min, and 85 °C for 5 min. cDNA was stored at -20 C. Real-Time quantitative PCR was performed with an iCycler IQ (BioRad Laboratories, Hercules, CA, USA).18S and GAPDH were used as an endogenous control to normalise the expression level of target genes. The primers were designed by using the software Beacon Designer 2.0 (PREMIER Biosoft International, Palo Alto, CA, USA). Primers used were listed in **(Table 1)**.

cDNA (25 ng) was amplified by PCR using primers for selected genes in separate wells with 1x iQ SYBR Green Supermix and 250nM gene-specific sense and antisense primers in a final volume of 25 µl for each well. The used iQ SYBR Green Supermix comprises of 100mM KCl, 40mM Tris–HCl, pH 8.4, 0.4mM each dNTP, 50 U/ml iTaq DNA polymerase, 6mM $MgCl_2$, SYBR Green I, 20nM fluorescein, and stabilizers. The PCR was performed in 96-well plates, each sample was performed in triplicate, and a no-template control was included for each amplification as a negative control. The thermal cycler conditions consisted of 3 min at 95 °C and 40 cycles at 95 °C for 20 s, 60 °C for 20 s and 72 °C for 30 s. In order to verify the specificity of the amplification, a melt-curve analysis was performed for; 1 min denaturation at 95 °C, 1 min annealing at 55 °C, and 80 cycles of 0.5 °C increments (10 s each) beginning at 55 °C. Thel melt-curve

analysis was carried out immediately after the amplification protocol. Non-specific products of PCR were not found in any case. A standard curve was generated using a "calibrator" cDNA (chosen among the cDNA samples), which was serially diluted and analysed. The iCycle iQ Real-Time PCR Detection System Software generated the equation describing the plots of the log10 of the starting quantity (micromoles) of 5 dilutions (125, 25, 5, 1, and 0.2 ng) of the calibrator cDNA versus the corresponding threshold cycle. The efficiency of qRT-PCR of primer pairs was calculated with the following formula:

Efficiency = $[10^{(-1/\text{slope})}] - 1$

To reduce the experimental variation, the results were normalised to 18S and GAPDH. The initial amount of the template of each sample was determined as relative expression versus one of the samples chosen as a reference (in this case the control sample) which is considered the 1x sample. Data were analysed using the Gene Expression analysis for iCycler V1.10 2004 (Bio-Rad). Briefly, for each set of replicates, the spreadsheet calculates the mean Ct and the standard deviation for the sample and derives a relative quantity from the equation:

Relative quantity = efficiency $^{(\text{control Ct} - \text{unknown Ct})}$

Where the control Ct is the Ct of a sample chosen as a control, and the unknown Ct is the value of the sample of interest. The normalisation factor is calculated as the geometric mean of the relative quantities for all reference genes use:

Normalization factor = (quant. ref.1* quant.ref.2*…* quant. ref.N) (1/N)

The unscaled expression level for the sample of interest is calculated using the equation:

Expression level = relative quantity/normalization factor

Then a scaled value dividing the unscaled expression level of each sample by the unscaled expression level of the control sample is calculated.

Protein extraction, quantification and SDS-PAGE: Liver and spleen tissues (50 µg) were thawed and homogenised with 150µl of 10mM HEPES (pH 7.4), containing 0.2mM $CaCl_2$ and 0.25M sucrose, using a tight Dounce–Potter device. Protein concentration was assessed spectrophotometrically with the bicinchoninic acid reaction with bovine serum albumin as standard [21].

SDS–PAGE separated proteins homogenate containing loading dye with 10% ß-mercaptoethanol in 10% acrylamide gel. Molecular weight standards 53-212 kDa (Amersham–Pharmacia, Buckinghamshire, UK) were used as marker proteins. The gel was run by electrophoresis as follow: 80V for stacking region and 180Vfor running region. Then, the gel was obtained and blotted onto a nitrocellulose membrane (PVDF) (Schleicher and Schuell, Dassel, Germany) with a wet blotting system (Sigma, St. Louis, MO, USA), using transfer solutions (700ml H2O, 200ml methanol 20%, and 100 ml transfer solution 10X) for 2 hour at 100V. After

transfer, the gel was checked by Coomassie blue and the PVDF membrane by red Ponceau. The membrane was washed three times with T-TBS (0.2% Tween 20; 20mM Tris; and 500mM NaCl; pH 7.5) and blocked with the 4% milkT-TBS solution for 1 hour at room temperature. The primary antibodies were diluted with the same blocking solution and incubated overnight at 4°C (1:2000 dilution). Next day the primary antibodies were removed, the membrane was washed and incubated with secondary antibodies for 1 hour at room temperature (1:5000 dilution). The peroxidase reaction was obtained by exposure of the membrane in the ECL-Plus Western Blotting detection system solutions (Amersham–PharmaciaBiotech, UK). After transfer to Kodak film, the bands were visualised by Kodak EDAS 290 (Kodak instruments, New Haven, Ct, USA) using Kodak 1D images software. The images were taken in TIFF formats and transferred to the Scion Image program. The optical density of the target proteins was taken and normalised to α-actin and represented as protein relative expression (ratio). List of antibodies and conditions of incubation were summarised in **(Table 2)**.

Statistical analysis: Data are reported as means ± SD. The two-tailed Student "t-test assessed differences between means". The values considered significant when $p<0.05$ *, $p<0.01$ ** and $p<0.001$ ***.

Results

Total serum bilirubin levels in β-thalassemic mouse and Gunn rat

TSB level was markedly elevated (6 folds) in β-thalassemic mouse compared to WT mouse, and in 17 days old jj Gunn rat (14 folds) compared to NN control rat **(Table 3)**.

MRP1 expression in the spleen of β-thalassemic mouse and liver of Gunn rat

Spleenic MRP1 mRNA and protein expression were expressed equally in both WT and β-thalassemic mice **(Figure 1A and 1B)**. Similarly, hepatic MRP1 mRNA and protein expression were not changed in jj Gunn rat compared to NN control rat at 17 days old **(Figure 2A and 1B)**.

MRP3 *expression in the spleen of β-thalassemic mouse and liver of Gunn rat*

Spleenic MRP3 mRNA expression was up-regulated (5.5 folds) in ß-thalassemic mouse compared to WT mouse. MRP3 protein was undetectable in WT mouse while 100% induced in β-thalassemic mouse **(Figure 3A and 3B)**. The C-18 antibodies recognise the two bands for MRP3 protein **(Figure 3A)**, which is probably the differentially glycosylated forms that were recognised by different antibodies used in the previous study [22]. Hepatic MRP3 mRNA and proteins were induced (3 folds and 2.5 folds, respectively) in jj Gunn rat than NN control rat 17 days old **(Figure 4A and 4B)**.

Discussion and conclusion

Unconjugated bilirubin (UCB) is responsible for neonatal jaundice and at this physiological condition UCB has significant cytoprotective roles. UCB accumulates in organs and causes cytotoxicity especially for neurons due to high blood concentration[6]. Because UCB can diffuse into any cell type, all cells must maintain low intracellular concentration; this is regulated by consumption (conjugation and oxidation) and/or export of the UCB back to blood serum[10]. The liver is the only organ that can conjugate UCB by the UGT1A1 enzyme and therefore other cell types should oxidise or export UCB to prevent its toxicity.

Previous studies focused on the possible role for MRP1 and MRP3 in exporting UCB out of the cells to prevent its intracellular toxicity. *In vitro* studies suggested that MRP1 might have a crucial role in protecting cells against UCB toxicity [11–14]. However, many of the published *in vitro* findings may be irrelevant to the *in vivo* condition [23]. Many factors may affect the *in vitro* studies such as the type of cell, species differences in the cellular systems employed, the length of exposure to UCB and cell age in culture. Several studies using *in vivo* and *in vitro* models also demonstrated an essential role for MRP3 in transporting UCB back to the bloodstream for renal excretion [15,16,24].

In the present work, the mRNA and protein expression profile for MRP1 and MRP3 were analysed *in vivo* under two different conditions of hyperbilirubinemia which was confirmed by measuring the TSB levels **(Table 3)**. Expression of both transporters was analysed in the spleen of the β-thalassemic mouse (chronic hemolysis - conjugated hyperbilirubinemia) and liver of Gunn rat (UGT1A1 deficiency – unconjugated hyperbilirubinemia) because each organ (with its related animal model) is exposed to high level of UCB.

In the spleen of the β-thalassemic mouse, qRT-PCR data and Western blot analysis indicated that UCB did not change the mRNA and protein expression of MRP1, while it induced the MRP3 mRNA expression **(Figure 1 and 3)**. Similarly, in the liver of Gunn rat, MRP1 mRNA and protein expression were not changed, while MRP3 mRNA and protein levels were induced by UCB **(Figure 2 and 4)**.

The results obtained in both animal models showed that any form of hyperbilirubinemia does not modulate MRP1expression *in vivo*. Analysis of MRP1 promoter constructs indicated that the promoter is TATA-less, CAAT-less and the GC rich may form the CpG island and suggested that MRP1 has a heavy promoter that makes this gene hardly to be induced under several conditions[25,26]. Furthermore, we cannot exclude any role for MRP1 (if any) in transporting UCB because the basal expression could be enough for its maximum transporting activity. Indeed, the mRNA basal expression for MRP1 is much higher than MRP3 in both tissues (data not shown).

The results also indicated that UCB induced the mRNA and protein expression of MRP3 transporter and suggested its possible role in exporting UCB back to the bloodstream. The induction of MRP3 by UCB seems to be at transcriptional level. Indeed, MRP3 gene was suggested to be an inducible form among MRPs family, and it has a promoter construct with TATA-box, CAAT-box, and has several enhancer cis-elements [27,28].

In conclusion, our *in vivo* study showed that the high level of UCB did not change MRP1 expression in the spleen of β-thalassemic mouse and liver of Gunn rat. MRP3 is induced in both organs exposed to high level of UCB. More *in vitro* experiments should be performed to analyse the direct role of MRP3 in preventing UCB toxicity

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Table 1. Primer sequences of genes designed for quantitative real time-PCR

Gene Name	*Accession number*	*Forward primers 5` - 3.` 5' to 3.'*	*Reverse primers 5` - 3.` 5' to 3.'*
MRP1	*NM_008576.*	*CGTGCTGTCCTGCTTCTC*	*CTTCTGATGTGTCCTCCTTATTC*
MRP3	*NM_029600*	*GGTTATCCAGGCTCAAGAC*	*GCCGTGGAAGGAATAGAAC*
*18S**	*NR_003278.*	*TCCGATAACGAACGAGAC*	*CTAAGGGCATCACAGACC*
*GAPDH**	*NM_008084.*	*CCAGTATGACTCCACTCACG*	*CTCGCTCCTGGAAGATGGTG*

*Housekeeping genes used to normalise the expression of target genes.

Table 2. Summary of antibodies

Target proteins	***1st antibodies***	***2nd antibodies***
MRP1	*A23 (Fernetti et al.,) 2001)*	*Goat anti-rabbit IgG/HRP (Dacko Lab.)*
MRP3	*C18(Santa Cruz Biotech)*	*Donkey anti-goat IgG/HRP (Jackson Lab.)*
α-actin*	*A2066 (Sigma Aldrich)*	*Goat anti-rabbit IgG/HRP (Dacko Lab.)*

*Housekeeping protein used to normalise the expression of target proteins.

Table 3: Total serum bilirubin (TSB) in WT and hyperbilirubinemic animal models

Animal	***Total serum bilirubin (TSB) mg/dl***
WT mouse	1.6.±.0.53
β-Thal mouse	5.95 ± 0.75
NN WT rat	0.33 ± 0.1
jj Gunn rat	13.83 ± 3.33

TSB was measured by diazo-reaction in five animals for each group

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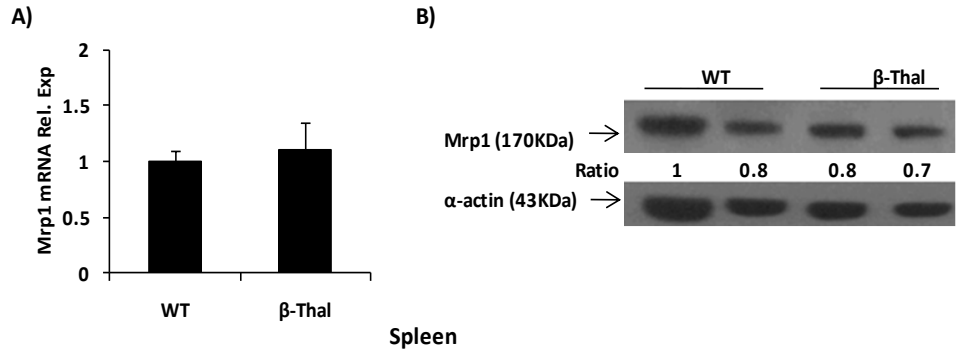

Figure 1: (A) MRP1 mRNA relative expression in the spleen of WT and β-thalassemic mouse. MRP1 expression was normalised to 18S and GAPDH and presented relative to WT controls**. (B)** MRP1 protein expression was analysed by western blot and normalised to α-actin then represented as a ratio.

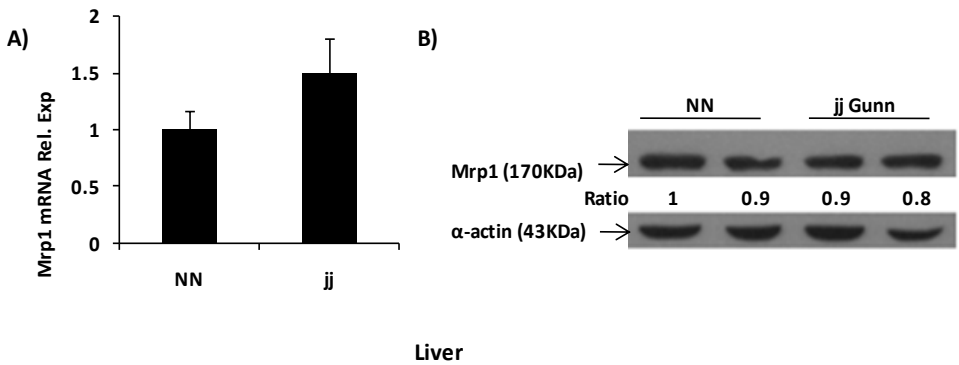

Figure 2: (A) MRP1 mRNA relative expression in liver of NN control rat and jj Gunn rat. MRP1 expression was normalised to 18S and GAPDH and presented relative to NN controls**. (B)** MRP1 protein expression was analysed by western blot and normalised to α-actin then represented as a ratio.

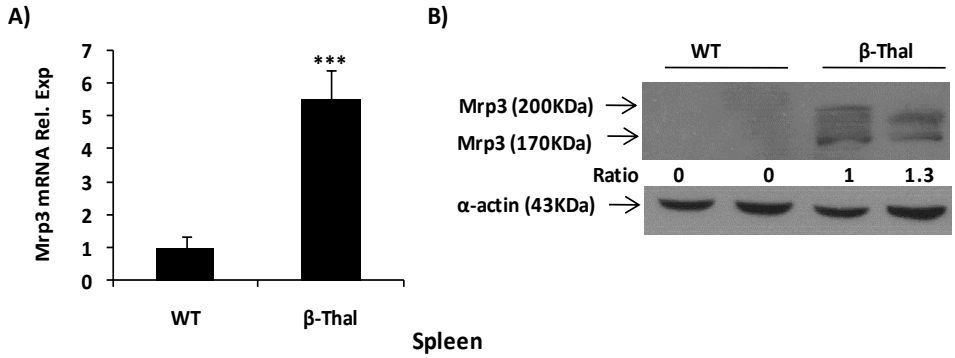

Figure 3: (A) MRP3 mRNA relative expression in the spleen of WT and β-thalassemic mouse. MRP1 expression was normalised to 18S and GAPDH and presented relative to WT controls**. (B)** MRP3 protein expression was analysed by western blot and normalised to α-actin then represented as a ratio.

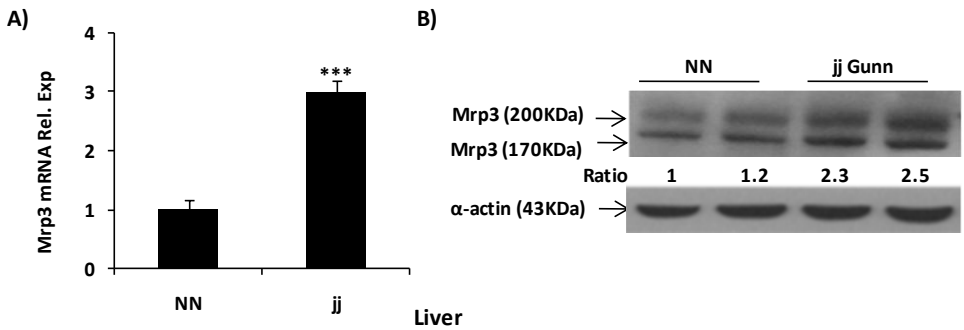

Figure 4: (A) MRP3 mRNA relative expression in liver of NN control rat and jj Gunn rat. MRP1 expression was normalised to 18S and GAPDH and presented relative to NN controls**. (B)** MRP3 protein expression was analysed by western blot and normalised to α-actin then represented as a ratio.